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REVIEW

D1-protein dynamics in photosystem II: the lingering enigma

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Abstract The D1/D2 heterodimer core is the heart of the photosystem II reaction center. A characteristic feature of this heterodimer is the differentially rapid, light-dependent degradation of the D1 protein. The D1 protein is possibly the most researched photosynthetic polypeptide, with aspects of structure–function, gene, messenger and protein regulation, electron transport, reactive oxygen species, photoinhibition, herbicide binding, stromal–granal translocations, reversible phosphorylation, and specific proteases, all under intensive investigation more than three decades after the protein's debut in the literature. This review will touch on some treaded areas of D1 research that have, so far, defied clear resolution, as well as cutting edge research on mechanisms and consequences of D1 protein degradation.

Keywords Photosynthesis · Protein turnover · Reaction center protein · Reactive oxygen species · *Spirodela oligorrhiza* · *Arabidopsis thaliana*

Abbreviations

Chl	Chlorophyll
PS	Photosystem
Q_A	The primary quinone electron acceptor in PS II
Q_B	The secondary quinone electron acceptor in PS II
PQ	Plastoquinone
Cyt b/f	Cytochrome b_6/f complex
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
PAR	Physiologically active radiation
UV	Ultraviolet

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Introduction

The photosystem II (PSII) reaction center in oxygenic phototrophs is dominated by the D1/D2 heterodimer core (Nanba and Satoh 1987). A characteristic feature of this heterodimer is the rapid, photon-flux-dependent catabolism of the D1 protein (Mattoo et al. 1984). D1 degradation in vivo is driven by a very broad, biologically relevant spectrum of radiance energy extending from UV-B, through UV-A, PAR, and into the far red. At least two photosensitizers are involved in mediating D1 breakdown, and concerted action of the two results in synergistic enhancement of degradation by a mechanism distinct from that involved under PAR or UV-B radiation alone (reviewed in Edelman and Mattoo 2006). During its short but eventful life history, the chloroplast-coded D1 precursor is C-terminally processed on the stromal lamellae following which it is translocated to the grana, where reversible phosphorylation and protein degradation occur (reviewed in Mattoo et al. 1999). Along the way, the D1 protein acts both as a structural, multifunctional component of the reaction center, mediating in a very direct way photosynthetic electron transport, oxygen evolution and reducing power (Aro et al. 2005; Barber 2006). Its rapid turnover is to some extent an outcome of its active life style, tied up in a still controversial way with photoinhibition (Adir et al. 2003; Yokthongwattana and

Melis 2006). Yet a majority of its degradation rate occurs at low light intensities and may be tied up with environmental regulation (Jansen et al. 1996) and signaling (Shlyk-Kerner et al. 2006). The D1 protein is possibly the most researched photosynthetic polypeptide, having overtaken even RUBISCO in Google hits. This review will touch on some treaded areas of D1 research that have, so far, defied clear resolution, as well as cutting edge research on the mechanism of D1 degradation.

Is singlet O_2 involved in D1 protein degradation?

It is well established that the oxidation of water and reduction of plastoquinone during the light reactions of photosynthesis cause an accumulation within PSII of oxidizing radicals, including reactive oxygen species (ROS) (Powles 1984; Asada 1996; Foyer and Noctor 2003; Apel and Hirt 2004). The rate of ROS accumulation in PSII increases with light intensity (Hirayama et al. 1995) and has been linked by many investigators with photoinhibition (reviewed by Adir et al. 2003), a process that reduces an organism's photosynthetic capacity as a result of excess light energy exceeding the ability for repair by PSII protein synthesis. Damage to the oxidizing side of the PSII reaction center via impaired electron donation from the oxygen-evolving complex (Callahan et al. 1986), and/or damage to the reducing side via blocked electron flow from Q_A^- to Q_B (Vass et al. 1992), was singularly, or sequentially (Song et al. 2006), implicated in eliciting ROS. Production of a Tyr radical (Barry and Babcock 1987), triplet chlorophyll (Takahashi et al. 1987), singlet oxygen (Setlik et al. 1990), superoxide anion (Liu et al. 2001), and hydroxyl radical (Pospíšil et al. 2004), were variously invoked in PSII inactivation.

PSII-derived ROS were theorized to trigger D1 protein degradation by changing the conformation of the protein and rendering it susceptible to protease (Aro et al. 1993). ROS were also suggested to act directly on the D1 protein, oxidizing amino acids close to the redox active components of PSII (Sharma et al. 1997). The idea that ROS may be involved in D1 degradation was based on evidence that scavengers of oxygen-free radicals such as propyl gallate and uric acid inhibit light-dependent degradation of the D1 protein while increasing the photosynthetic efficiency of *Spirodela* plants (Sopory et al. 1990). The involvement and mechanism of ROS action in photoinhibition via effects on D1 degradation and/or synthesis remains an active area of research (Okada et al. 1996; Trebst et al. 2002; Mizusawa et al. 2003; Nishiyama et al. 2004; Takahashi and Murata 2008).

Among ROS, singlet O_2 ($^1O_2$) has recently received added attention, in particular as a signaling molecule and damaging species during photoinhibition (Trebst et al. 2002, 2004; Skovsen et al. 2005). However, Sopory et al. (1990) argued against the involvement of $^1O_2$ in degradation of the D1 protein in vivo because D_2O, an effective stabilizer of $^1O_2$ (Merkel et al. 1972), actually increased the half-life of D1, while selenomethionine, a scavenger/quencher of $^1O_2$ (Tappel 1965), had no effect. Sopory et al. (1990) concluded that other ROS, such as hydroxy radicals, might cause damage to D1 that results in its degradation; since then, hydroxyl radical generation by PSII has been reported (Pospíšil et al. 2004). These results notwithstanding, studies linking $^1O_2$ and D1 metabolism persist. Loss of D1 at photoinhibitory light intensities was correlated to endogenous loss of the antioxidant α-tocopherol (Trebst et al. 2002). Based on this correlation, Kreiger-Liszkay and Trebst (2006) concluded that: "it is singlet oxygen that induces D1 protein degradation, as concluded earlier from other experimentation…. but sometimes still in doubt."

Is $^1O_2$ really involved in D1 degradation during photoinhibition? Murata and coworkers have proposed an alternative explanation for the involvement of ROS in the damage of the PSII reaction center (Nishiyama et al. 2004; Takahashi and Murata 2008). They suggest that ROS ($^1O_2$ as well) are involved in inhibiting the synthesis of D1 and therefore affect the repair cycle. Based on this, we hypothesize that repair processes, involving to a major extent D1 synthesis (Adir et al. 2003; Yokthongwattana and Melis 2006), are predominant features in situations where plants can protect against photoinhibition. Thus, antioxidants and oxygen-free radical scavengers should, in fact, provide for robust chloroplast protein synthesis, including D1 synthesis, and enhance PSII electron transport machinery. Indeed, this has been observed in our work with *Spirodela* plants. Propyl gallate, the free-oxygen scavenger, strongly promotes D1 synthesis (by 3.5–5.8-fold) and results in higher photosynthetic efficiency compared to untreated control plants (Sopory et al. 1990).

In order to unambiguously attribute plant responses to endogenous $^1O_2$ production, there is a need to quantify $^1O_2$ in vivo. This has now been made possible by a Singlet Oxygen Sensor Green (SOSG) reagent, which is selective for $^1O_2$ with non-interference from hydroxy or superoxide radicals (Molecular Probes 2004; Flors et al. 2006). *Arabidopsis* leaves at growth light intensity (150 μmol m^{-2} s^{-1}) produced small amounts of $^1O_2$, which increased considerably at higher (600 μmol m^{-2} s^{-1}) light intensity (Flors et al. 2006). Leaves were also painted with 200 μM DCMU, incubated up to 90 min at a light intensity of 350 μmol m^{-2} s^{-1} and then returned to darkness for 12 h. SOSG fluorescence was observed within 30 min of light exposure, and SOSG fluorescence, recorded at 90 min, persisted for at least 12 h in the dark (Flors et al. 2006). These authors concluded that in *Arabidopsis*, $^1O_2$ is produced both

in the dark and in the light and is further enhanced in a DCMU treated leaf.

The data of Flors et al. (2006) bring a new perspective and question the current understanding of the role of $^1O_2$ in PSII function, in particular D1 degradation and photoinhibition. First, $^1O_2$ production at light intensities lower than 150 μmol m^{-2} s^{-1} is minimal, however at this fluence, the D1 degradation rate in *Spirodela* has already passed the 50% mark (see Fig. 1). At fluences lower than 150 μmol m^{-2} s^{-1}, for example at 6 μmol m^{-2} s^{-1}, where significant *Spirodela* D1 degradation occurs (Greenberg et al. 1989a; Sopory et al. 1990; Jansen et al. 1993), $^1O_2$ production is expected to be barely of consequence. Second, DCMU treatment produces $^1O_2$ at levels (Flors et al. 2006) that can be quite detrimental to PSII. However, it is well established that D1 degradation is blocked in the presence of DCMU (Mattoo et al. 1984). In light of these new observations (Flors et al. 2006) and our previous studies (Sopory et al. 1990), the involvement of $^1O_2$ in D1 degradation is questionable and remains unresolved.

It is important to appreciate that at low to moderate light fluences plants and algae synthesize relatively abundant amounts of antioxidants, such as tocopherol, ascorbate, and glutathione in their photosynthetic compartments to protect macromolecules and cellular machinery against oxidative damage (Foyer et al. 2006; Kreiger-Liszkay and Trebst 2006). Not surprisingly, therefore, tocopherol biosynthesis responds to oxidative stress, including high light fluences in higher plants (Torres et al. 1989; Munne-Bosch and Alegre 2002; Havaux et al. 2005a) and cyanobacteria (Backasch et al. 2005), and to moderate fluences in algae (Kreiger-Liszkay and Trebst 2006).

D1 protein degradation in vivo is mainly associated with low photon fluences

Based on in vitro (Ohad et al. 1985; Arntz and Trebst 1986; Jegerschöld et al. 1990) and in vivo (Schuster et al. 1988; Aro et al. 1993; Sundby et al. 1993) studies, D1 protein degradation has been associated in the minds of many investigators with photoinhibitory radiance intensities supersaturating for photosynthesis (reviewed by Adir et al. 2003; Yokthongwattana and Melis 2006). This approach reached its apex in studies that measured D1 degradation in vitro at intensities up to 7000 μmol m^{-2} s^{-1} (Jegerschöld et al. 1990), approximately an order of magnitude above the light saturation point for many photosynthetic organisms.

However, in fact, D1 reaction center protein degradation is a process largely associated with low fluences in the intact plant (Mattoo et al. 1984). This was definitively demonstrated in a large study by Jansen et al. (1999), who followed degradation of the D1 protein in *Spirodela* plants

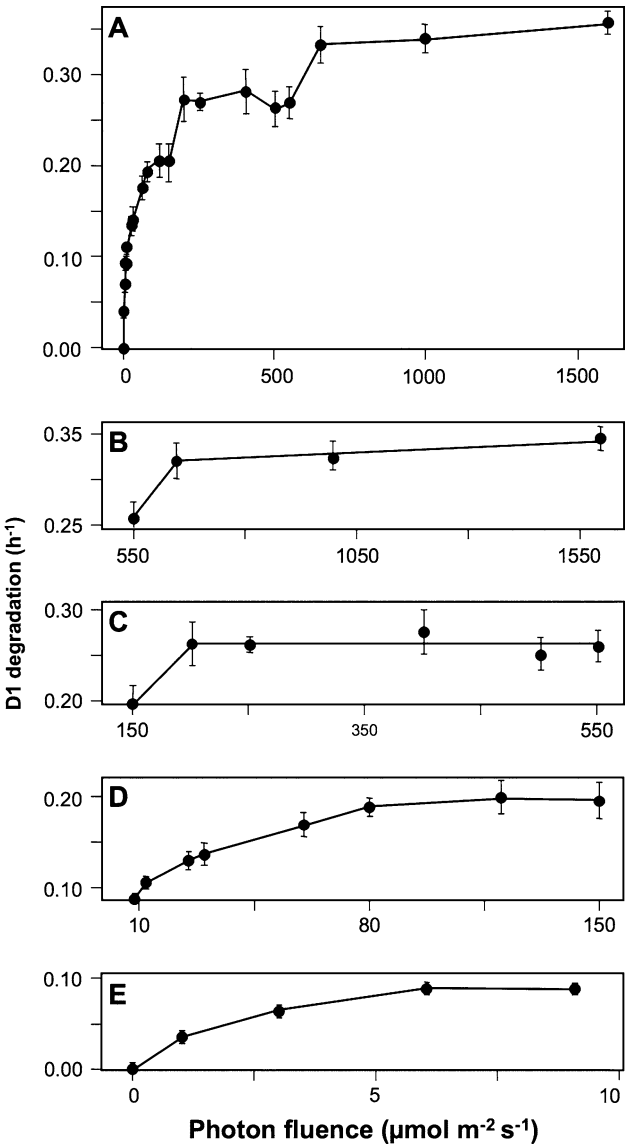

Fig. 1 Complex rate saturation kinetics for D1 protein degradation in vivo: *Spirodela* plants were radiolabeled with ^{35}S-methionine for 2 h at 25 μmol m^{-2} s^{-1} of PAR and chased in growth medium containing 1 mM non-radioactive methionine at the photon fluences indicated. D1 Protein degradation was measured as the disappearance of the 32 kDa band following SDS-PAGE. Quantification was by microdensitometry, and the data normalized internally in each gel lane to the level of the stable light-harvesting chlorophyll a/b protein band (Greenberg et al. 1987). The identity of bands was periodically checked by immunoblotting. $n = 14$–30 for each photon fluence point, with the total number of gel lanes analyzed = 400. Standard errors of the mean are shown. All calculations of kinetics were based on data points taken within the first two half lives of the D1 protein. A *t*-test revealed highly significant (≥99%) deviations from monophasic logarithmic saturation curve at the critical fluence ranges of 3–90 μmol m^{-2} s^{-1}, 80–250 and 400–650 μmol m^{-2} s^{-1}. (**A**) overall response from 0 to 1,600 μmol m^{-2} s^{-1}; (**B–E**) extended view of individual phases: *Phase I*, from 0 to 10 μmol m^{-2} s^{-1}; *Phase II*, from 10 to 150 μmol m^{-2} s^{-1}; *Phase III*, from 150 to 550 μmol m^{-2} s^{-1}; *Phase IV*, from 550 to 1,600 μmol m^{-2} s^{-1}. (adapted from Jansen 1993)

as a function of photon fluence rate at 20 intensities, over a range extending from 6 μmol m^{-2} s^{-1} to 1,600 μmol m^{-2} s^{-1}, the equivalent of full sunlight. A fluence as low as 6 μmol m^{-2} s^{-1} elicited a reaction constituting more than 25% of the total degradation response, while a fluence of 200 μmol m^{-2} s^{-1} triggers more than 75% of the total response. In agreement, using pulse-chase methodology, it was demonstrated that in *Brassica* leaves D1 protein degradation is mainly associated with low (<350 μmol m^{-2} s^{-1}) photon fluences (Sundby et al. 1993). Likewise, using immunodetection, D1 degradation in *Chlamydomonas* cells treated with a chloroplast protein synthesis inhibitor was demonstrated to be significantly associated with low photon fluences (Keren et al. 1995).

The connection between light intensity, photoinhibition and D1 protein degradation was directly addressed by Tyystjärvi and Aro (1996) who defined photoinhibition as "light-dependent irreversible inactivation of PSII reaction center activity, which can be restored only via the degradation and synthesis of the Dl protein." Analyzing pumpkin leaves in the presence of a chloroplast protein synthesis inhibitor, the authors proposed that photoinhibition in vivo occurs monolithically at all light intensities and by one dominant mechanism. This was based on the observations that: the apparent quantum yield of photoinhibition remained constant under all photon flux densities in their experiments, degradation of the Dl protein in low light depended on photon flux density in the same way as did photoinhibition, and there was kinetic agreement between immunodetected Dl protein degradation and the inactivation of PSII. Thus, it was concluded that the turnover of the Dl protein depends on photoinhibition even in low light. Moreover, citing the absence of two-photon events in their study, the authors argued that acceptor side photoinhibition does not contribute to photoinhibition in vivo.

While this orderly picture of D1 protein degradation and its relationship to photoinhibition and PSII reaction center activity is fetching, we find several problems. First, our calculations of the rather dispersed 9 μmol m^{-2} s^{-1} measurement data in Fig. 3c and d of Tyystjärvi and Aro (1996), upon which the orderly picture partially rests, show weak linear correlations ($R^2 < 0.3$), raising doubts concerning the low light intensity effects. Second, Keren et al. (1995) showed that the apparent quantum yield for D1 protein degradation in *Chlamydomonas* cells is not constant, but rather several folds higher at low fluences than that observed at intensities inducing photoinactivation of the PSII reaction center. Third, D1 protein degradation in *Spirodela* plants at physiological light intensities can proceed in the absence of PSII electron transport as measured by Q_B-mediated electron flow, and vice versa (Jansen et al. 1990). Fourth, the in vivo evidence for D1 protein degradation in *Spirodela* consistently points to acceptor side, rather than donor side, damage. Thus: (1) D1 protein degradation in vivo at low photosynthetic fluence rates is inhibited by diuron (Mattoo et al. 1981, 1984), atrazine (Sopory et al. 1990), and substituted nitrophenols (Jansen et al. 1993) that interact with specific regions of the Q_B niche on the acceptor side of the PSII reaction center. (2) The initial 23.5 kDa D1-breakdown fragment in vivo was shown by proteolytic mapping to arise from scission of the protein chain between helices D and E, on the acceptor side of the reaction center (Greenberg et al. 1987) under a wide spectrum of radiances (Greenberg et al. 1989b). (3) The in vivo action spectrum for UV-B driven D1 degradation matches the absorbance spectra for the various states of plastoquinone (Greenberg et al. 1989a), which resides in the Q_B niche on the acceptor side.

The photon fluence response curve for D1 protein degradation is unexpectedly complex

Lastly, while many biological processes increase linearly in rate as a function of stimulant concentration until saturation, the photon fluence response curve for D1 protein degradation in vivo is decidedly non-linear and unexpectedly complex. In *Spirodela* plants, an extensive study (Jansen et al. 1999) showed at least four distinct degradation rate phases consisting of separate rises in rate followed by flat plateaus when going from darkness to the equivalent of full sunlight (Fig. 1). Each phase comprised 20–30% of the total response (Fig. 1B–E). Close inspection of the light-dependent D1 degradation described for *Chlamydomonas* (Keren et al. 1995) also reveals a multiphasic trend, such that complex D1-degradation photo-saturation-kinetics is likely to be a general characteristic of oxygenic phototrophs and needs an explanation. Indeed, there is no lack of speculations: It is possible that multiphasic kinetics reflect the existence of distinct populations of PSII reaction centers (Neale and Melis 1991) characterized by differences in functional stability (Anderson et al. 1997). Alternatively, it is known that the D1 protein undergoes a number of post-translational modifications during its life cycle (Edelman and Mattoo 2006), among them, phosphorylation, which saturates at *Phases II* and *III* fluences (Rintamäki et al. 1997), and de-phosphorylation, which saturates at the very low fluences of *Phase I* (Elich et al. 1993). A correlation was also proposed between degradation of the D1 protein at *Phase I* light intensities and the generation of QB-/S2,3 states (Keren et al. 1995; Keren et al. 1997), while at the high end of the fluence scale, donor or acceptor side inactivation in the intact plant correlates with D1 degradation at photoinhibitory *Phase IV* light intensities (Barber and Andersson 1992).

A multiplicity of D1-specific degradation proteases

D1 protein degradation continues to elicit extensive attention in the literature. Major advances have recently been made in identifying the proteases that degrade this rapidly turning over PSII core protein, and in unraveling the mechanisms of proteolytic action. Can the D1 proteases help to explain the complex D1 degradation kinetics? We think so.

Two families of proteases have emerged as major players in catabolism of mature D1 protein: the ATP-independent serine endoprotease Deg/Htr family (Huesgen et al. 2005) and the ATP-dependent zinc metalloprotease FtsH family (Adam et al. 2006). *Arabidopsis thaliana* contains 16 Deg genes and 12 FtsH genes. Four Deg proteases were experimentally localized to the chloroplast (Huesgen et al. 2005), with Deg1, Deg5, and Deg8 attached to the luminal side of the thylakoid membrane (Itzhaki et al. 1998), and Deg2 attached peripherally on the stromal side (Haußühl et al. 2001). Four FtsH proteases, FtsH1, FtsH2, FtsH5, and FtsH8, have likewise been experimentally identified in the thylakoid proteosome (Friso et al. 2004) following molecular genetic analysis of leaf variegation mutants (Sakamoto et al. 2002; Bailey et al. 2002), whose loci encode FtsH 2 and FtsH5. Similar to their *E. coli* counterparts, the plastidic Deg (Chassin et al. 2002; Sun et al. 2007) and FtsH proteases (Sakamoto et al. 2003) are active in monomeric and especially hexameric form. The FtsH hexamers were further shown to be heteromeric (Yu et al. 2004), containing 'Type A' (FtsH1, FtsH5) and 'Type B' (FtsH2, FtsH8) subunits. Mutant studies showed that the presence of at least one protein from each type is essential for FtsH hexamers to accumulate and function (Zaltsman et al. 2005). FtsH1, FtsH2, and FtsH5 were found as integral proteins in the thylakoid membrane, with their ATP binding domain and catalytic zinc-binding site facing the stroma (Lindahl et al. 1996).

Stroma-side proteases

The ATP independence, sub-membranal location and endoproteolytic activity of Deg2 dovetail nicely with the requirements for D1 protein degradation in *Spirodela* plants at low light intensities. Thus, disappearance of the 32 kDa D1-protein band in *Spirodela* plants pulse-chased at 40 µmol m^{-2} s^{-1} was independent of energy from photophosphorylation (Mattoo et al. 1984), while appearance of a radiance-dependent N-terminal 23.5 kDa degradation product, cleaved in the stroma-located DE-loop (Fig. 2) between residues 238 and 347 of the D1 protein, was demonstrated at 15 µmol m^{-2} s^{-1} (Greenberg et al. 1987) and 6 µmol m^{-2} s^{-1} (Greenberg et al. 1989b) PAR in pulse-labeled plants. In a second step, the 23.5 kDa degradation product was itself rapidly degraded (Greenberg et al. 1987). In illuminated spinach thylakoids, ATP-independent accumulation of the N-terminal 23 kDa fragment was found to be GTP-dependent while proteolysis of the 23 kDa primary fragment itself was shown to be ATP- and zinc-dependent (Spetea et al. 1999). As in the in vivo *Spirodela* study, generation of the primary cleavage product was the rate-limiting step. The chloroplast protease responsible for stroma-side cleavage in the DE-loop was later identified in *Arabidopsis* as Deg2 (Haußühl et al. 2001). FtsH protease, the only known ATP- and zinc-dependent protease in thylakoid membranes, was shown in *A. thaliana* to be involved in the secondary degradation of the 23 kDa fragment, a reaction that can occur in isolated thylakoids incubated in the dark (Lindahl et al. 2000).

Besides the stroma-exposed DE-loop, the N-terminus of the D1 protein (Fig. 2) is an additional region that has been implicated as a stroma-side determinant for rapid D1 degradation. Removal of 20 residues from the N-terminal tail of D1 in *Synechocystis* 6803 by mutagenesis produced a strain that could assemble oxygen-evolving dimeric PSII complexes but accumulated D1 precursor protein and showed inhibited D1 protein degradation and PSII repair at high light intensities (Komenda et al. 2007). The phenotype

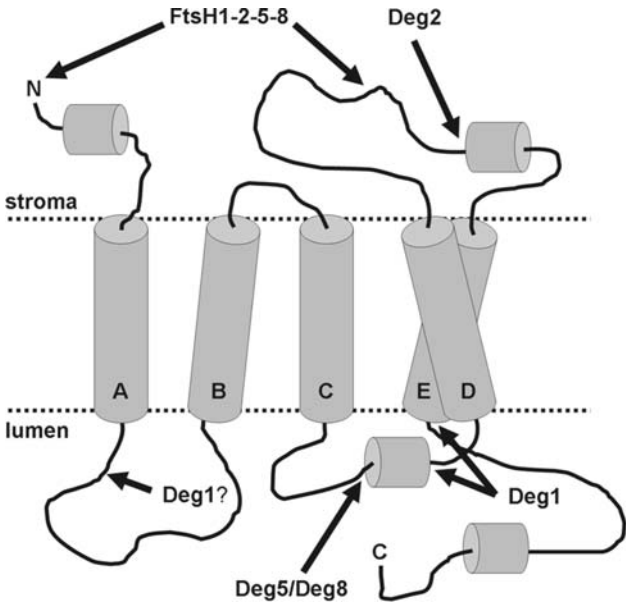

Fig. 2 A model for proteolytic degradation of the D1 protein: A cartoon representation is shown based on the crystal structure of photosystem II from *Thermosynechococcus elongatus* (Loll et al. 2005). The membrane spanning helices of the D1 proteins and the amino and carboxy termini are indicated. The shorter parallel helices in the CD-loop, DE-loop, and amino and carboxy extensions are also drawn. Approximate sites of cleavage by FtsH and Deg proteases as described by Kapri-Pardes et al. (2007), Sun et al. (2007), and Komenda et al. (2007) are shown, taking in account the preference for cleavage by Deg proteases after Val or Ile (Kolmar et al. 1996)

of this strain was very similar to that of *ftsH* null mutants impaired in D1 degradation (Silva et al. 2003), thus FtsH was invoked as the protease involved.

Lumen-side proteases

Following initial publication of the N-terminal 23.5 kDa breakdown product in vivo at low light intensities (Greenberg et al. 1987), a number of in vitro studies with isolated thylakoid membranes or PSII core particles appeared in which a 23–24 kDa degradation product of the D1 protein was found following application of strong photoinhibitory light. PSII particles from wheat leaves yielded both N- and C-terminal 23–24 kDa fragments of the D1 protein, identified by specific antibody probes (De Las Rivas et al. 1992). A C-terminal fragment appeared when an electron acceptor was present and the water splitting reaction was not functional, suggesting cleavage of the D1 protein in the AB-loop on the luminal side (Fig 2). When the water splitting reaction was active and an electron acceptor was not added, an N-terminal 23 kDa D1-protein fragment was detected, and cleavage at the QEEET motif in the DE-loop on the stroma side was suggested. Under both conditions, 10 kDa fragments complementary to the 23–24 kDa ones were also seen, as was a 16 kDa fragment (De Las Rivas et al. 1992).

Recent studies in *Arabidopsis* have identified the proteases involved in lumen-side rapid degradation of D1 as Deg1 (Kapri-Pardes et al. 2007) and Deg5 and Deg8 (Sun et al. 2007). C-terminal 16 kDa and 5.2 kDa D1-fragments were significantly reduced in amount in a mutant containing reduced levels of the Deg1 protease. When recombinant Deg1 was applied to inside-out mutant thylakoid membranes (with the Deg 1-depleted lumen now exposed to the medium) formation of the 5.2 kDa fragment was induced (Kapri-Pardes et al. 2007). Based on fragment size, cleavage in the CD-loop downstream of the parallel CD-helix, and cleavage immediately downstream of helix E were invoked for the 16 and 5.2 kDa fragments, respectively (Fig. 2). The differential abundance of the 16 and 5.2 kDa fragments suggested that the former underwent further cleavage and degradation. Similarly, it was demonstrated that turnover of newly synthesized D1 protein in a deg5–deg8 double mutant of *Arabidopsis* was significantly impaired versus wild type (Sun et al. 2007). Recombinant Deg8 was proteolytically active toward photodamaged D1 protein, producing 16 kDa N-terminal and 18 kDa C-terminal fragments, suggesting cleavage of the CD-loop in vivo just upstream of the parallel CD-helix (Fig. 2). Finally, the cleavage site in the AB-loop that was reported early on (De Las Rivas et al. 1992) is hypothesized to result from Deg protease degradation as well (Kapri-Pardes et al. 2007).

Knockout mutants

The composite picture of a battery of identified proteolytic enzymes acting to degrade the D1 protein that emerged from the in vivo and in vitro studies received a jolt with the publication of protease knockout experiments. Triple knockouts of all three Deg protease homologs in *Synechocystis* 6803 showed continued, even accelerated, degradation of the D1 protein in pulse-chase experiments under high light treatment without detection of breakdown products. These results were interpreted as showing that the Deg proteases are not obligatory for D1 degradation and PSII repair in vivo (Barker et al. 2006). Analogously, the rate of D1 protein degradation under light stress conditions in *Arabidopsis* knockouts lacking the Deg2 protease were found to be similar to those in wild-type plants (Huesgen et al. 2006). The authors concluded that the primary cleavage of photodamaged D1 protein within the DE-loop by Deg2 protease, as demonstrated earlier by them in vitro under light stress conditions (Haußühl et al. 2001), is not a prerequisite for its degradation in vivo. These results, along with those from the N-terminal D1 protein deletion mutant (Komenda et al. 2007), spawned a model for proteolytic degradation of the D1 protein in vivo, that is driven solely by FtsH proteases (Komenda et al. 2007). The far-reaching conclusion was proffered that under 'normal' conditions, FtsH-mediated N-terminal proteolysis selectively replaces damaged D1 without producing detectable breakdown products (Nixon et al. 2005).

Interplay among variety, radiance and intensity

While Tyystjärvi and Aro (1996) concluded that stromal acceptor-side photoinhibition and its concomitant D1 protein degradation seen in vitro essentially do not occur in vivo, Nixon et al. (2005) concluded that D1 degradation in vivo is, normally, exclusively driven by stroma-side proteolysis. We do not support either conclusion, because too much of the accumulated data need to be excluded to allow either one. Rather, as put forward by others (Huesgen et al. 2006; Kapri-Pardes et al. 2007; Sun et al. 2007), we conclude that several D1 protein degradation pathways exist in vivo. To quote Kapri-Pardes et al. (2007): "The results accumulated to date suggest that degradation of the D1 protein should be viewed as a two-step process: single cleavage events at hydrophilic regions of the protein on both sides of the thylakoid membrane, by Deg1, Deg2, and possibly other peptidases, to yield a limited number of distinct fragments, followed by their complete proteolysis by the processive ATP-dependent FtsH protease".

We propose that a key to understanding some of the conflicts and nuances in D1 protein degradation lies in the

multi-phasic photo-fluence degradation-rate kinetics shown in Fig. 1. The different degradation-rate phases may represent the effects of various D1-specific proteases 'kicking in' at increasing light intensities. Considering only data from in vivo experiments in which degradation fragments were shown, the primary D1 protein cleavages at the luminal CD-loop associated with Deg1 and Deg5–Deg8 would require no more than moderate and high light stimulations, respectively (Kapri-Pardes et al. 2007; Sun et al. 2007). There are no comparable data available for the primary cleavage at the lumenal AB-loop. On the stromal side, the primary cleavage at the DE-loop by Deg2 would require low light stimulation (Greenberg et al. 1987), while stroma-side N-terminal cleavage attributed to FtsH protease would require no more than moderate light stimulation (Komenda et al. 2007). While the degradation-rate kinetics curve (Fig. 1, upper panel) can be interpreted biochemically and physiologically in several fashions, one simple way to interpret the data is to assume that once an enzyme has reached its photon flux threshold it continues to operate at higher photon flux densities as well. As a result, the overall proteolytic effect on degradation will be largely additive. This interpretation is attractive as it potentially resolves many discrepancies between studies. Thus, continued degradation of the D1 protein, measured as the loss of the 32 kDa band on a radiogram or immunodecorated blot following gene knockout, can result from the continued activity of one degradation-rate phase or another in the face of inhibition of some others. Thus, elevated degradation of the D1 protein in the triple Deg knockouts of *Synechocystis* 6803 (Barker et al. 2006) need not imply that Deg proteases are gratuitous for D1 degradation under 'normal' conditions (Nixon et al. 2005). It more likely means that at the high photoinhibitory light intensities employed (Barker et al. 2006), the damaged D1 protein is a sufficient substrate for the combined family of FtsH proteases, acting as an exoproteinase (Chiba et al. 2002) and an endoproteinase (Okuno et al. 2006), to degrade D1 in an atypical way, maybe in association with other plastid proteases that are up-regulated in response to high light stress (Sinvany-Villalobo et al. 2004). Similar arguments can be made for the Deg 1 (Kapri-Pardes et al. 2007), Deg 2 (Huesgen et al. 2006), and Deg5-Deg8 (Sun et al. 2007) knockouts. Likewise, mutants with extensive deletions in the DE-loop that show elevated degradation of D1 protein at moderate light intensities as measured by loss of the 32 kDa band (Nixon et al. 1995; Mulo et al. 1997), need not imply that the deleted loop regions are uninvolved in wildtype D1 degradation. Instead, it can be understood that both lumenal-side Deg and stroma-side FtsH proteolyses continue to act in the mutants, resulting in the disappearance of the 32-kDa band. In other words, knockout mutant results need not imply that processive N-terminal FtsH proteolysis is the main stay of normal D1 protein degradation and that all other D1 proteolyses operate minorially, or under abnormal or unusual stress conditions (Komenda et al. 2007).

Will the primary cleavage site for D1 please stand up!

The reader will have noticed that different D1-protein degradation studies claim different 'primary' cleavage products. It is likely that all the claims are correct, because the various proteolytic events in D1 degradation need not occur sequentially. Rather, they most likely happen simultaneously (Kapri-Pardes et al. 2007). As a result, FtsH would need to extract no more than a single transmembrane helix at a time from the membrane, a feat demonstrated for FtsH in *E. coli* membranes (Kihara et al. 1999).

Regulatory functions for D1-protein primary degradation fragments?

Perhaps the most intriguing aspect of D1 protein degradation is the possibility that the degradation fragments serve not only as a source for plastid amino acid pools and numerous research articles, but also have a defined biological function. A recent preliminary report (Stelljes and Koenig 2007) now raises the possibility that primary degradation fragments of D1 serve important regulatory roles in the D1 life cycle. There are 3 *psbA* genes in *Synechococcus elongatus* 7942 coding for the D1 protein. Expression of *psbAI* dominates at low light intensities and is down regulated under high light in favor of the other two genes, which are then induced. Electrophoretic mobility shift assays showed that the untranslated leader of *psbAI* message binds one or more unidentified proteins from an *S. elongatus* extract (Nair et al. 2001). Stelljes and Koenig (2007) now claim that D1 degradation products, originating from the carboxy two thirds of the protein (fragments B2 and B3), may be the unidentified polypeptides. The resulting hypothesis is that the D1 protein regulates its own resynthesis during light-dependent turnover by its cleavage products. Moreover, fragment B3 (covering the carboxy precurser end through the DE-loop) shows a high binding affinity for a sequence 0.8 to 1.3 kb upstream of *psbAI* showing similarity to *isiB*, a gene coding for flavodoxin, which when derepressed, can substitute for ferredoxin under oxidative and high-light stress (Havaux et al. 2005b), and may be involved in cyclic electron transport around photosystem I (PSI) in addition to ferredoxin (Hagemann et al. 1999). While the evidence from Stelljes and Koenig (2007) is preliminary, indirect (potentially occurring D1 degradation products were overexpressed in *E. coli* and

protein-DNA interaction with the promoter and upstream regions of *psbAI* then assayed) and needs verification, the possible involvement of D1 in transcriptional regulation of PSI would close a cycle concerning D1 protein phosphorylation: PSII regulates phosphorylation of D1 (Elich et al. 1992), PSI regulates dephosphorylation of D1 (Elich et al. 1993), D1 fragments regulate PSI (still hypothetical).

The role of phosphorylation in the D1 protein life cycle

D1 is synthesized as a 33.5–34 kD precursor (Grebanier et al. 1978; Edelman and Reisfeld 1978; Reisfeld et al. 1982) on chloroplast ribosomes anchored on the non-appressed stromal lamellae (Mattoo and Edelman 1987). There, it is processed at the carboxy terminus (Marder et al. 1984) as a part of the maturation process. C-terminal processing of precursor D1 is required for the assembly of the manganese-cluster into PSII (Diner et al. 1988; Satoh and Yamamoto 2007). Mature D1 assembles into a native PSII complex on the stromal lamellae (Ghirardi et al. 1990, 1993) and then translocates to grana lamellae (Mattoo and Edelman 1987), where it is functional. At the granal lamellae, D1 is reversibly phosphorylated (Elich et al. 1993) at the N-terminal threonine in a redox-regulated manner (Michel et al.. 1988; Elich et al.. 1992). These spatio-temporal events in the life history of the D1 protein relate to the configurational and functional heterogeneity of PSII (Anderson and Melis 1983; Callahan et al. 1989; Lavergne and Briantais 1996).

In vivo pulse-chase experiments with ^{32}P-orthophosphate (Elich et al. 1992, 1993; Koivuniemi et al. 1995; Booij-James et al. 2002) or ^{35}S-methionine (Callahan et al. 1990), followed by fractionation of thylakoid membranes into non-appressed stromal and appressed grana lamellae (Callahan et al. 1989) were used to demonstrate that phosphorylation of D1, and degradation of D1 and its phosphorylated form, occur on granal lamellae. The identity of phosphorylated D1 was confirmed by immunoprecipitation and immunoblotting (Callahan et al., 1990; Elich et al. 1992; Booij-James et al. 2002) using antibodies against D1, while the purity (98%) of the stromal and granal lamellae fractions was ascertained by ultrastructural and cytochemical characterization of the isolated lamellae and determination of Chl a/b and CPI/CPII ratios (Callahan et al. 1989; Ghirardi et al. 1993).

The conclusion that degradation and dephosphorylation of D1 occur on the grana is at variance with reports from the laboratory of E-M Aro that phosphorylated D1 migrates to stromal lamellae and dephosphorylates prior to D1 degradation (Baena-Gonzalez et al. 1999; Rokka et al. 2000). However, we note that the latter interpretations on intramembranal movement of phosphorylated D1 mostly relied on immunoblot data using a generic commercial polyclonal antibody to phospho-threonine (Bergo et al. 2002). Further, the ultrastructural purity and biochemical characteristics of the isolated grana and stromal lamellae were not described. This is of particular significance in light of the findings on macroscopic rearrangements and dynamic behavior of thylakoid architecture involving fission and fusion at the interface of stromal and grana lamellae (Chuartzman et al. 2008). A critical assessment of data presented in the literature on D1 phosphorylation using anti-phosphothreonine antibodies reveals inconsistencies and variations in signal [for instance, compare Fig. 1 in Pursiheimo et al. (2003) with other figures in that paper]. It is important to ascertain that such antibodies do in fact immunoprecipitate a ^{32}P-labeled D1. In studies to be published elsewhere (Krol M, Ivanov AG, Booij-James IS, Mattoo AK and Huner NPA, unpublished data), immunoblots with a commercial phosphothreonine antibody (Zymed Labs., Inc., USA), employed also by the Aro laboratory (Baena-Gonzalez et al. 1999; Rokka et al. 2000; Bergo et al. 2002; Pursiheimo et al. 2003), showed a cross reaction with a putative 'phosphorylated D1' protein band in wild type barley leaves but not in the *Chlorina F2* mutant. However, when in vivo and in vitro radiolabeling studies were conducted, respectively with ^{32}P-orthophosphate and γ–^{32}P-ATP, it was demonstrated that the D1 protein is actually phosphorylated in both the wild type and *F2* barley mutant.

Surprisingly, the role of phosphorylation in D1 degradation has remained stubbornly unclear. Light-dependent D1 protein phosphorylation appears to occur universally in higher plants, but not in the moss *Ceratodon purpureus* (Rintamaki et al. 1995a), or in the green alga *Chlamydomonas* (Delepelaire and Wollman 1985), or in cyanobacteria (Allen 1992). Phosphorylation of D1 was variously suggested to be involved in: prevention of PSII disassembly (Aro et al. 1992), promotion of PsbH protein dissociation from the PSII core (Giardi 1993), regulation of the repair cycle of photoinhibited PSII centers (Rintamaki et al. 1995b), or controlling the timing of D1 proteolysis under photoinhibitory conditions (Aro et al. 2005). However, Booij-James et al. (2002) showed that D1 phosphorylation in *Spirodela* is regulated by a circadian clock, with the greatest amount of D1 phosphorylation occurring significantly before maximal diurnal light intensity both under photoinhibitory and low light conditions. Moreover, using a molecular genetics approach, Bonardi et al. (2005) reported that reversible D1 phosphorylation is not essential for PSII repair in *Arabidopsis*. Thus, the involvement of phosphorylation in D1 degradation has yet to be satisfactorily resolved.

Perhaps light-mediated reversible phosphorylation is a means for the chloroplast to anticipate environmental change (Booij-James et al. 2002). The manner in which D1 metabolic regulation is achieved will differ among photosynthetic

organisms. In higher plants, where D1 is reversibly phosphorylated, circadian regulation of metabolism may be at the phosphorylation level. In cyanobacteria, where redox-regulated phosphorylation of D1 does not occur, different D1 isoforms adapted in vivo to different photon radiances (Bustos et al. 1990; Clarke et al. 1993; Kulkarni and Golden 1994) do the job. We thus propose a generalized hypothesis, whereby reversible phosphorylation of D1 (and other PSII proteins) in higher plants evolutionarily replace multiple DNA copies in cyanobacteria as a more energy efficient substrate for regulation of PSII core metabolism.

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